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Fluorescence Correlation Spectroscopy at Micromolar Concentrations: Shattering the Concentration Barrier

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Abstract

Fluorescence Correlation Spectroscopy (FCS) is an important technique for studying biomolecular interactions dynamically that may be used *in vitro* and in cell-based studies. It is generally claimed that FCS may only be used at nM concentrations. We show that this general consensus is wrong and that the limitation to nM concentrations is not fundamental, but due only to detector limits and laser fluctuations. With a high count rate detector system and laser fluctuation corrections, we demonstrate FCS measurements up to 38 μM with the same signal to noise as at lower concentrations.

Fluorescence Correlation Spectroscopy is an important technique for the dynamic measurement of interactions between molecules in biological systems[1,2]. Molecules diffusing through a small optical detection volume, defined using confocal microscopy or sometimes super-resolution microscopy[3,4], lead to fluctuations in fluorescence intensity. By calculating correlation functions from these fluctuating signals, information on concentration and diffusion rates of the molecules is obtained. The amplitude of the resulting correlation function is inversely proportional to the concentration of the fluorescent molecules, and the timescale of the correlation decay is related to diffusion rates. Binding to other molecules or structures may be detected as changes in diffusion rates. Additionally, if the binding partners are labeled with two different colors, amplitudes from cross-correlation calculations indicate binding[5]. This class of methodologies has been widely used both *in vitro* and in cell-based studies.

FCS measures dynamics using the intensity correlation $g^{(2)}(\tau)$ of fluorescence, which is calculated according to the formula,

$$g_I^{(2)}(\tau) = \langle I(t) I(t+\tau) \rangle \Big/ \langle I(t) \rangle^2 \tag{1.1}$$

For a single diffusing species in a Gaussian detection volume of lateral width ω and longitudinal width *l*, the correlation function can be modeled using

$$g_I^{(2)}(\tau) = 1 \Big/ \left[N\left(1+\tau/\tau_D\right) \sqrt{1+\left(\omega/l\right)^2 \tau/\tau_D} \right], \tag{1.2}$$

where *N* is the molecular occupancy of the Gaussian detection volume (the average number of molecules in the detection volume – proportional to concentration) and $\tau_D = \omega^2/4D$ where *D* is the diffusion coefficient. Most often, experimental data are fitted sufficiently well to the simplified model,

$$g_I^{(2)}(\tau) = 1 \Big/ \left[N\left(1+\tau/\tau_D\right) \right]. \tag{1.3}$$

Since the correlation amplitude decreases with increasing concentration, it is intuitive to think that the signal to noise on the measurement would decrease with increasing concentration. However, the earliest statistical analysis of FCS indicated that the signal to noise of the measurement depended only on the fluorescence intensity *per molecule*, *q* (often called molecular brightness), and was independent of concentration [6], citing reference [7] as experimental validation. This would mean that there is no fundamental upper limit on the concentration range possible for use with FCS. Further calculations better accounting for photon statistics and lower concentrations again found that, as the concentration increases to infinity, *q* remains the critical parameter and the signal to noise is independent of concentration[8]. Statistical accuracy was calculated for the modern confocal detection geometries, obtaining similar results[9]. Reference [9] additionally experimentally verified the independence of the correlation function signal to noise on concentrations up to 200 nM, and emphasized that increasing *q* only helps the signal to noise up to the point where molecular diffusion noise dominates.

Despite these longstanding indications of the possibility of using FCS at much higher concentrations, nearly all FCS publications that comment on this issue indicate that FCS may only be used at low, nanomolar concentrations [2–4,10,11]. Here, we show using simulations and experiments that FCS may be

used at micromolar concentrations as long as the detectors can handle the high count rates and other sources of fluctuations, especially laser excitation fluctuations, are corrected.

In order to determine the behavior of FCS accuracy at higher concentrations, we performed simulations of fluorescent molecules diffusing through a Gaussian detection volume with averages of 0.1, 1, 10, 100, and 1000 molecules inside the detection volume at one time. The simulation box is 180 times larger than the detection volume by volume, and the diffusion time was 2.5 ms. For each simulation, 180,000 molecules were simulated for 10 seconds with 1 μs time steps. Figure 1(a) shows normalized time traces (divided by mean count rate) for the 10 s simulations with N = 0.1, 10, and 1000. The dramatic decrease in the relative size of the fluctuations leads to the intuitive, but incorrect, conclusion FCS would have low signal to noise at high concentrations. Normalizing the time traces differently by subtracting the mean and dividing by the standard deviation, the fluctuations FCS measure are revealed in Figure 1(b). The fitted values for N and τ_D are shown in Figure 1(c), and examples correlations for different values of N are shown in Figures 1(d)-(f). The results in Figure 1 demonstrate that the errors on the fitted diffusion times and molecular occupancies (concentration) do not increase with increasing concentration, supporting the original analysis of signal to noise [6,8,9].

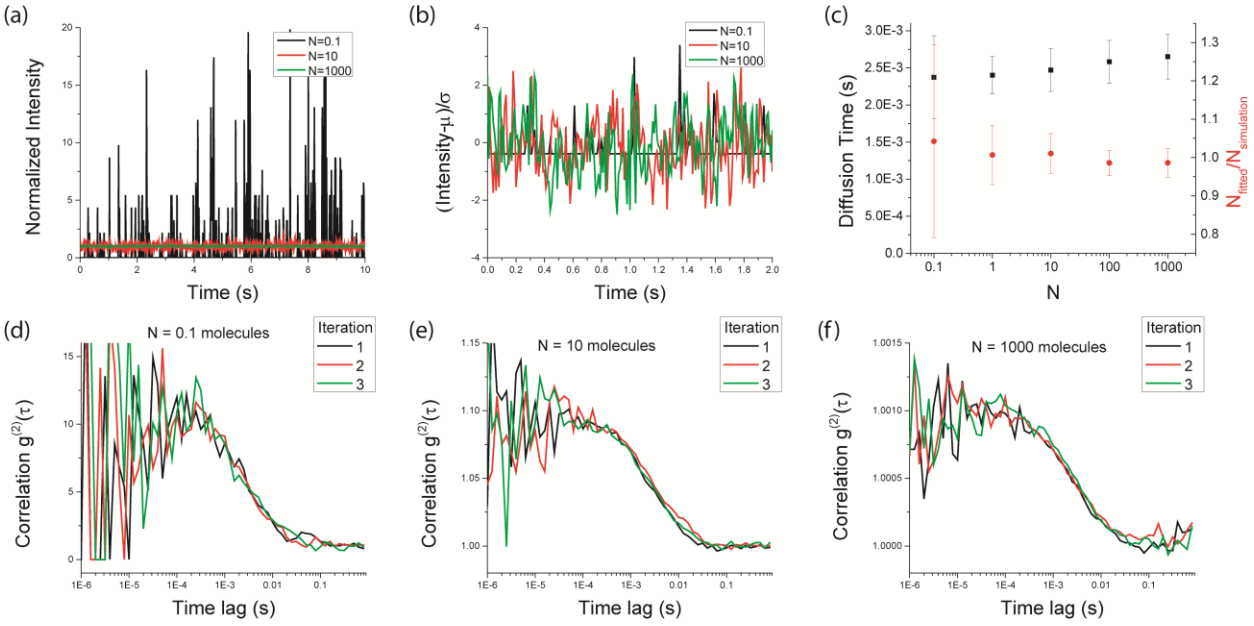

Figure 1: Simulations of diffusing fluorescent molecules in a Gaussian detection volume show that the accuracy of FCS is constant with increasing concentration, quantified as N, the molecular occupancy of the detection volume. **(a)** Example time traces for N=0.1, N=10, and N=1000 are shown, normalized by dividing by the mean intensity. **(b)** Subtracting the mean and dividing by standard deviation of time trace intensities from part (a), the fluctuations that FCS measures are revealed. **(c)** The errors on fitted diffusion times and concentrations for the calculated correlations do not decrease with increasing concentration. Left axis is diffusion time and right axis is the ratio of the fitted occupancy of detection volume (concentration) divided by the simulation value (from x axis). Error bars are calculated using 30 repeated simulations. Correlations for simulated 10 s measurements of fluorescent molecules diffusing through solution are shown in **(d)** for N=0.1, **(e)** for N=10 and **(f)** for N=1000.

Given that the signal to noise of FCS measurements does not decrease with increasing concentration based on statistical analysis and simulations, why have no experiments shown that it is possible to perform FCS at much higher concentrations than the nanomolar limits frequently cited? There are two important constraints. First, in order to retain the signal to noise on FCS correlations, it is necessary to keep the intensity per molecule q constant. This means that, if the signal to noise is to remain the same, increasing the concentration by a factor of 10 increases the total detected count rate by a factor 10. For very high concentrations, detector saturation limits the amount of signal that may be measured. If the experimenter reduces laser excitation to avoid saturation, the signal to noise of the FCS measurement decreases rapidly. A second limitation arises from the fact that the relative fluctuations at high concentrations are small. Although the signal to noise does not change for ideal experiments, other fluctuations, especially those from laser excitation variations, have correlations that can obscure the fluctuations we want to observe. Neither of these limitations is fundamental, and can be overcome with proper technological improvements and modifications.

The experimental setup and data analysis for this work are shown schematically in Figure 2. A home-built confocal microscope with single molecule sensitivity is used [12,13]. A stable 487 nm diode laser (Stradus 488, Vortran Laser) is coupled through an optical fiber and used for excitation. We have used two sets of photodetector "banks", each consisting of four APD detectors (PD-050-CTD, Micro-Photon-Devices) to increase the count rates available. Additionally, a photodiode (PDA-155, Thorlabs) is used to monitor the laser power. Previously, a photodiode was used to correct for laser fluctuations, but the methodology for applying the correction was different and was not used in the context of high concentration FCS[14]. The 8 channels of photon counts and laser monitor channel are acquired simultaneously using two synchronized data acquisition cards as 500 kHz (PCIe-6351, National Instruments). We used a photon counting strategy rather than photon timing to avoid saturating the data acquisition system with the high count rates. The measurements were performed on free Alexa 488 dye with concentrations calibrated using a spectrophotometer. We use a 1.4 NA oil immersion objective and a 75 μm pinhole. Our fitted molecular occupancy values indicate a detection volume of around 1.8 fl.

Modeling the measured signal M as the product of two independent random processes I (fluorescence) and L (laser intensity), the correlation of M is the product of the correlations of I and L:

$$g_M^{(2)}(\tau) = g_I^{(2)}(\tau) g_L^{(2)}(\tau), \tag{1.4}$$

so that correcting for laser fluctuations can be accomplished using the following equation:

$$g_I^{(2)}(\tau) = g_M^{(2)}(\tau) / g_L^{(2)}(\tau) \tag{1.5}$$

In order to calculate the correlation functions $g_M^{(2)}(\tau)$ and $g_L^{(2)}(\tau)$ from our measurements, a cross-correlation approach was used to eliminate spurious detector correlations. Cross-correlation of the two detector banks was used to calculate $g_M^{(2)}(\tau)$ and cross-correlation of the laser monitor photodiode and one of the detector banks was used to calculate $g_L^{(2)}(\tau)$. Equation (1.5) was then used to obtain the corrected fluorescence correlation function, $g_I^{(2)}(\tau)$. The results of this calculation are illustrated in Figures 2(b) and 2(c), where the correlation due to laser fluctuations is removed from the total correlation

function by division. In calculating these correlations, it is necessary to account for the deadtimes of the APDs in order to properly remove the laser fluctuations. The correlations are calculated using a modification of the multi-tau algorithm[15]. The primary change is that, for each bin j, a corrected intensity $I_j = n_j / \left(t_j - n_j \Delta t\right)$ is used for correlation calculations rather than the number of photon counts n_j. t_j is the width of the bin over which n_j is acquired, and Δt is the dead time for each photodetector. Ensuring the linearity of the photodetectors or correcting for any non-linearity accurately is critical for Equation (1.5) to work well.

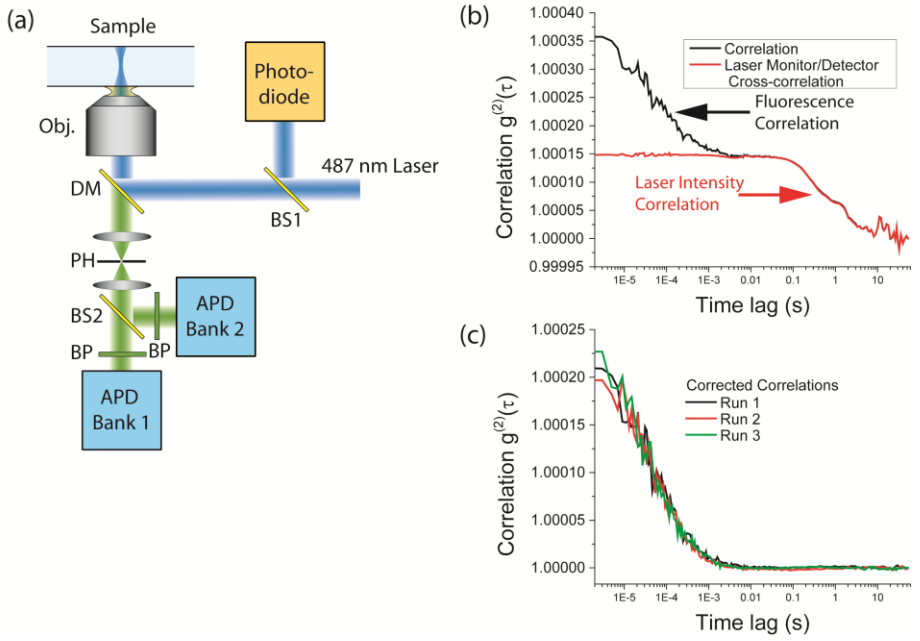

Figure 2: Experimental setup for measuring FCS at micromolar concentrations. **(a)** Emission from a confocal microscope is monitored by two APD banks of 4 APD photodetectors each. A photodiode simultaneously monitors the laser intensity. **(b)** A cross-correlation of the signal from the two APD banks is shown as the black line. The cross-correlation between one APD bank and the photodiode, which only contains contributions from laser intensity fluctuations, is shown in red. **(c)** Division of the two correlations in part (b) leads to corrected correlations. Three examples of measurements with 3.8 µM Alexa 488 are shown.

We performed two series of measurements with constant laser power but varying concentration to demonstrate FCS at µM concentrations. By keeping the laser power constant for a series of concentrations, we demonstrate that the signal to noise of the correlations and values extracted from them do not vary within the measurement error. For the measurements made with 20 µW laser power, 4 one-minute measurements were made at each concentration shown in Table 1. For those with 5 µW, 8 one-minute measurements were made at each concentration. By fitting the resulting corrected correlations to the model in Eq. (1.3), we extracted molecule occupancy N and diffusion time τ_D, and quantified the goodness of fit using χ^2. The mean and standard deviation of the fitted values for each sample and laser power are shown in Table 1. For 5 µW laser power, we were able to go to higher concentrations (up to 38

μM) before saturation. Figure 3 shows the averaged corrected correlations for the 8 minutes of measurements made on free Alexa 488 fluorophores for the 5 μW laser power. Figure 3(d) shows these correlations after normalization using the fitted molecular occupancy *N*. The overlap of these normalized correlations, the consistency of the mean and standard deviation of the fitted diffusion times and molecular occupancies in Table 1 verify that, fundamentally, FCS signal to noise remains constant for arbitrarily high concentrations. Only detector saturation and other sources of fluctuations obscure the correlation signal.

	5 μW Laser Power			**20 μW Laser Power**		
Sample	**38 μM**	**3.8 μM**	**380 nM**	**3.8 μM**	**380 nM**	**38 nM**
N	41300 ± 1300	4600 ± 300	380 ± 40	4900 ± 100	348 ± 4	33.5 ± 0.5
τ_D (μs)	57 ± 5	60 ± 5	54 ± 10	57 ± 2	55 ± 1	54 ± 1
χ^2	1.0	1.0	1.2	1.7	2.0	2.0

Table 1: Fitted values for molecular occupancy *N* (proportional to concentration), diffusion time (τ_D) and fit quality (χ^2) for various concentrations of Alexa 488 and two laser powers. The mean and standard deviation of fits for 8 measurements at 5 μW and 4 measurements at 20 μW of 1 minute each are shown.

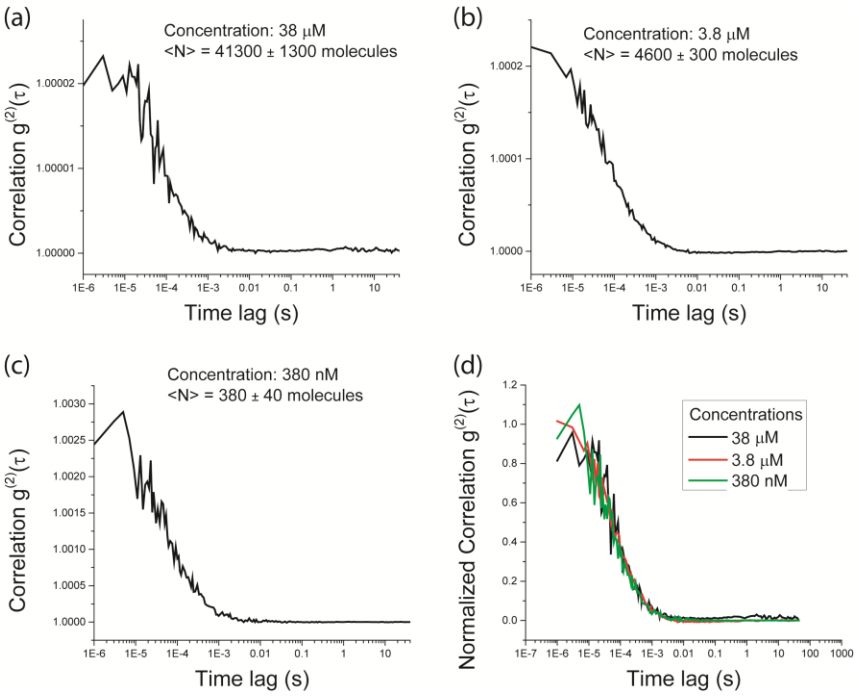

Figure 3: Example correlation curves for Alexa 488 at three different concentrations with constant laser power of 5 μW: **(a)** 38 μM, **(b)** 3.8 μM, and **(c)** 380 nM. **(d)** Correlations normalized using fitted molecular occupancy have similar noise and overlap.

In conclusion, with proper detectors and corrections, FCS may be performed at arbitrarily high concentrations. We have demonstrated FCS at up to 38 μM concentrations with our current experimental setup, and expect further increases in possible concentrations with future improvements in detectors. We

attempted measurements at 380 μM, but the laser power had to be decreased too far (400 nW) to allow for experiments in a reasonable time. Nano-confinement or superresolution approaches to increasing the concentration range of FCS are not necessary for this purpose. Nano-confinement is still necessary for isolation of single molecules at higher concentration[3] and performing FCS over varying sub-diffraction detection volume sizes[16]. This approach may be easily combined with those approaches and with cross-correlation measurements. The results of this work greatly enhance the potential impact of FCS for monitoring molecular interactions both *in vitro* and in living cells.

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